Eur. J. Biochem. ■, ■–■ (1996)
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The identification of 11β-hydroxysteroid dehydrogenase as carbonyl reductase of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

Edmund MASER[1], Elmar RICHTER[2] and Jutta FRIEBERTSHÄUSER[1]

[1] Department of Pharmacology and Toxicology, School of Medicine, University of Marburg, Germany
[2] Walther-Straub Institute of Pharmacology and Toxicology, Ludwig-Maximilians University of Munich, Germany

(Received 22 January/29 March 1996) – EJB 96 0080/1

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which is generated by nitrosation of nicotine, requires enzymatic activation by cytochrome-*P*450-mediated α-carbon hydroxylation to yield particularly powerful carcinogenic alkylating intermediates. Pyridine-N-oxidation and carbonyl reduction are detoxification pathways, the latter by providing the functional hydroxy moiety necessary for glucuronosylation and final excretion of NNK. For more than a decade, the enzyme responsible for NNK carbonyl reduction has awaited characterization. In the present study, we demonstrate that the NNK carbonyl reductase is identical to 11β-hydroxysteroid dehydrogenase (11β-HSD), the physiological function of which is the oxidoreduction of glucocorticoids. We conclude that the expression of 11β-HSD (together with glucuronosyl transferase) may have profound influence on the carcinogenic potency of NNK and that many compounds of endogenous and exogenous origin, which are known to be substrates or inhibitors of 11β-HSD, may modulate NNK-induced carcinogenicity by competing for the same enzyme. In light of the known species and tissue differences in the expression of 11β-HSD isozymes, important aspects of NNK-induced tumorigenesis, such as metabolic activation versus inactivation or organospecificity, can now be re-evaluated.

Keywords: 11β-hydroxysteroid dehydrogenase; glucocorticoid metabolism; carbonyl reduction; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; tobacco-specific nitrosamines.

Tobacco smoke is a complex mixture of more than 4000 substances (Roberts, 1988), among which at least 40 have been identified as carcinogens, tumor initiators, or promoters in laboratory animals (International Agency for Research on Cancer, 1986). Tobacco-specific nitrosamines, which are formed by nitrosation of nicotine during maturation and air-curing of tobacco, as well as during combustion of cigarettes, represent the most abundant and potent carcinogens in tobacco products and tobacco smoke (Hecht and Hoffmann, 1988; Hoffmann and Hecht, 1985), and convincing evidence for their role as causative factors in several adult human cancers has been discussed (Hoffmann and Hecht, 1985; Hecht, 1994; Preston Martin, 1991).

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been identified as one of the strongest nitrosamine carcinogens in tobacco products (Rivenson et al., 1988), with relatively high concentrations (Tricker et al., 1991). NNK requires metabolic activation in order to exert its carcinogenic effect (Fig. 1). This activation involves the α-hydroxylation of either the α-methylene or methyl carbon of NNK, resulting in the formation of electrophiles which can methylate and pyridyloxobutylate, respectively, hemoglobin and DNA (for review cf. Hecht, 1994).

Correspondence to E. Maser, Department of Pharmacology and Toxicology, School of Medicine, University of Marburg, Lahnberge, D-35033 Marburg/Lahn, Germany

Fax: +49 6421 285600.

Abbreviations. 11β-HSD, 11β-hydroxysteroid dehydrogenase; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

Enzyme. 11β-Hydroxysteroid dehydrogenase (EC 1.1.1.146).

Reduction of the carbonyl group of NNK to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is a very efficient pathway of NNK metabolism in animals and human tissues (Liu et al., 1990; Jorquera et al., 1992; Murphy et al., 1990; Schulze et al., 1995; Guo et al., 1992; Demkowicz-Dobrzanski and Castonguay, 1992; Morse et al., 1990b; Rossignol et al., 1989). NNAL, like NNK, can possibly also be α-carbon-hydroxylated to alkylating intermediates (Castonguay et al., 1983). Detoxification pathways include pyridine-N-oxidation of both NNK and NNAL as well as glucuronosylation of NNAL (Hecht, 1994).

Recent studies have demonstrated the involvement of several cytochrome *P*450 enzymes in the α-carbon hydroxylation and pyridine N-oxidation of NNK (Hecht, 1994; Smith et al., 1992a). However, although constituting the major pathway of NNK metabolism in several tissues (Liu et al., 1990; Jorquera et al., 1992; Murphy et al., 1990; Schulze et al., 1995; Guo et al., 1992; Demkowicz-Dobrzanski and Castonguay, 1992; Morse et al., 1990b; Rossignol et al., 1989), the enzyme which catalyzes the carbonyl reduction of NNK to NNAL in microsomal fractions has not been identified until now.

11β-Hydroxysteroid dehydrogenase (11β-HSD; EC 1.1.1.146) is a microsomal enzyme responsible for the interconversion of active 11-hydroxyglucocorticoids to inactive 11-oxo forms (Monder and Shackleton, 1984). By this action 11β-HSD protects the non-selective mineralocorticoid receptor from exposure to cortisol or corticosterone, and modulates access of glucocorticoids to glucocorticoid receptors (Edwards et al., 1988; Funder et al., 1988). It is generally accepted that there are at least two isoforms of 11β-HSD in mammals, a ubiquitous low-

FEBS Eur. J. Biochem. Ms.-No. 756 (96 0080/1) Ms. 1-31 Pages 1-6
Springer-Verlag, 69042 Heidelberg 1 / Wiesbadener Graphische Betriebe GmbH, 65050 Wiesbaden
Provisorische Seitenzahl / Provisional page number S11ag75756 1. Korr. Date

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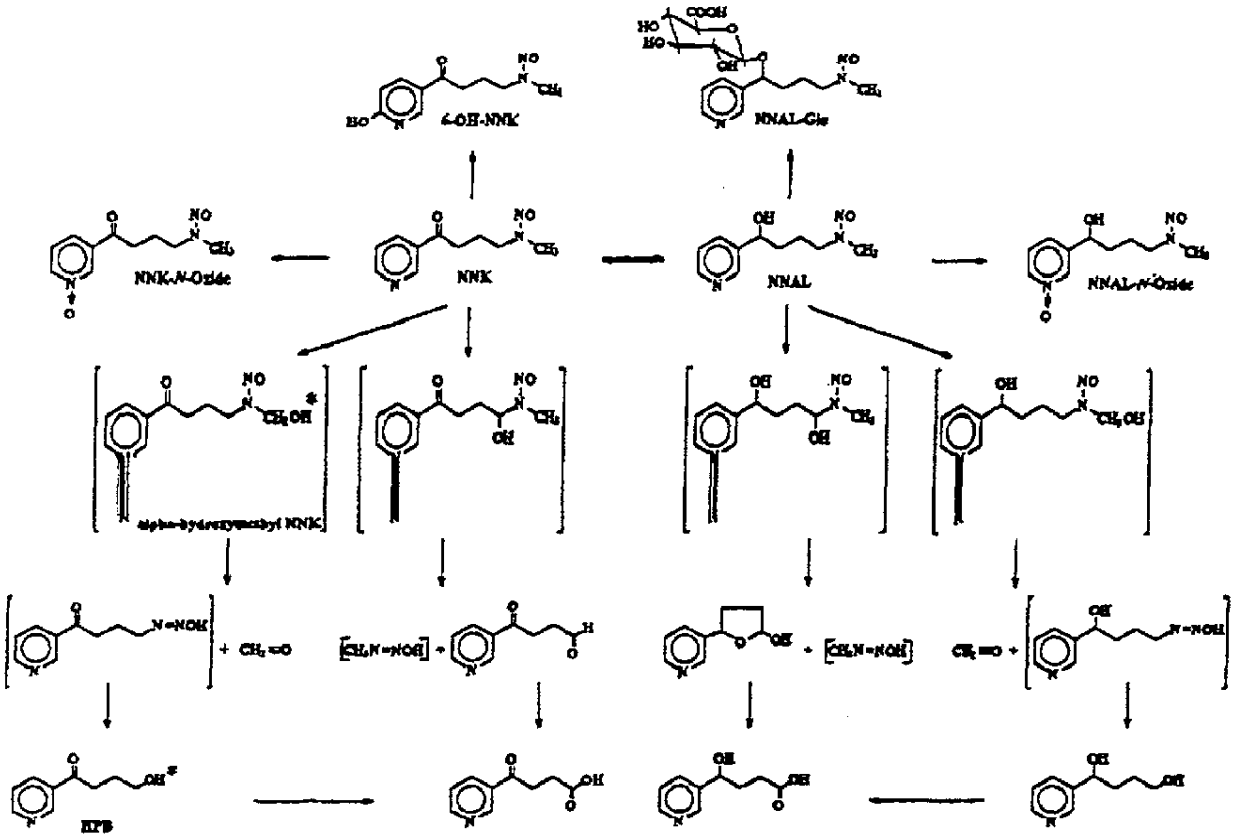

Fig. 1. NNK metabolism. Recently, glucuronosylation of α-hydroxymethyl NNK and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), respectively, at the positions indicated by an asterix has been demonstrated (Murphy et al., 1995).

affinity $NADP^+$-dependent enzyme (11β-HSD1) (Maser and Bannenberg, 1994a; Oppermann et al., 1995) and a tissue-specific high-affinity NAD^+-dependent form (11β-HSD2) (Albiston et al., 1994). Both isoforms belong to the short-chain dehydrogenase superfamily of enzymes. However, sequence alignment revealed only 14% identity between 11β-HSD 1 and 11β-HSD 2 (Albiston et al., 1994).

In previous investigations we have shown that the liver-type 11β-HSD (11β-HSD1) is a pluripotent enzyme in that it is capable of catalyzing the reduction of several non-steroidal xenobiotic carbonyl compounds, in addition to being specific towards its physiological steroid substrates (Maser and Bannenberg, 1994b). In this study we demonstrate that 11β-HSD from mouse liver acts as carbonyl reductase for the tobacco-specific nitrosamine NNK, and by this action initiates the final inactivation of NNK via glucuronosylation.

MATERIALS AND METHODS

Animals. Livers of female NMRI mice (25–30 g) were used as source for the purification of 11β-HSD. Animals were kept in the Department of Pharmacology and Toxicology (Marburg) on a standard laboratory pellet diet and water *ad libitum* at a fixed 12-h light/dark cycle starting at 06:00 h.

Chemicals. Enzymatic tests were performed using NADPH, $NADP^+$, Glc6*P* and Glc6*P* dehydrogenase from Boehringer Mannheim. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was purchased from Campro Scientific (Emmerich, Germany). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) was a gift from D. Hoffmann (American Health Foundation, Valhalla NY). Glucocorticoids (corticosterone and 11-dehydrocorticosterone), glycyrrhetinic acid as well as low-molecular-mass markers were purchased from Sigma. Peroxidase-conjugated anti-(rabbit IgG) antibodies, which were used as secondary antibodies, were from Dakopatts (Hamburg). All other chemicals used were of either HPLC or reagent grade and were obtained from Merck.

Purification of 11β-hydroxysteroid dehydrogenase (11β-HSD). The purification of 11β-HSD for enzymatic assays and for immunization was performed as described previously (Maser and Bannenberg, 1994a).

Immunization and preparation of antisera. Immunization and preparation of antisera against 11β-HSD of mouse liver was performed as described elsewhere (Maser and Bannenberg, 1994b).

Affinity purification of antibodies against 11β-HSD. Purified 11β-HSD protein was loaded onto SDS gels, electrophoresed and blotted as described elsewhere (Maser and Bannenberg, 1994b). After blocking of unspecific binding sites, nitrocellulose sheets were incubated overnight with anti-(11β-HSD) antisera (1:500) in NaCl/Tris pH 7.4 containing 1% fetal calf serum, and washed three times with NaCl/Tris for 15 min. Dissociation of 11β-HSD-specific antibodies was achieved by washing the membrane with 100 mM glycine/HCl pH 2.5 for 5 min. The resulting affinity purified supernatant containing anti-(11β-HSD) antibody was removed, brought to pH 7.5 with 0.4 M Tris, lyophilized and stored at −80°C. For immunoinhibition studies, antibodies were resolved in 10 mM sodium phosphate pH 7.4.

Assay of NNK carbonyl reduction by 11β-HSD. The assay of NNK carbonyl reduction was performed by incubating 10 µl of purified mouse liver 11β-HSD (corresponding to 1.9–5.2 µg

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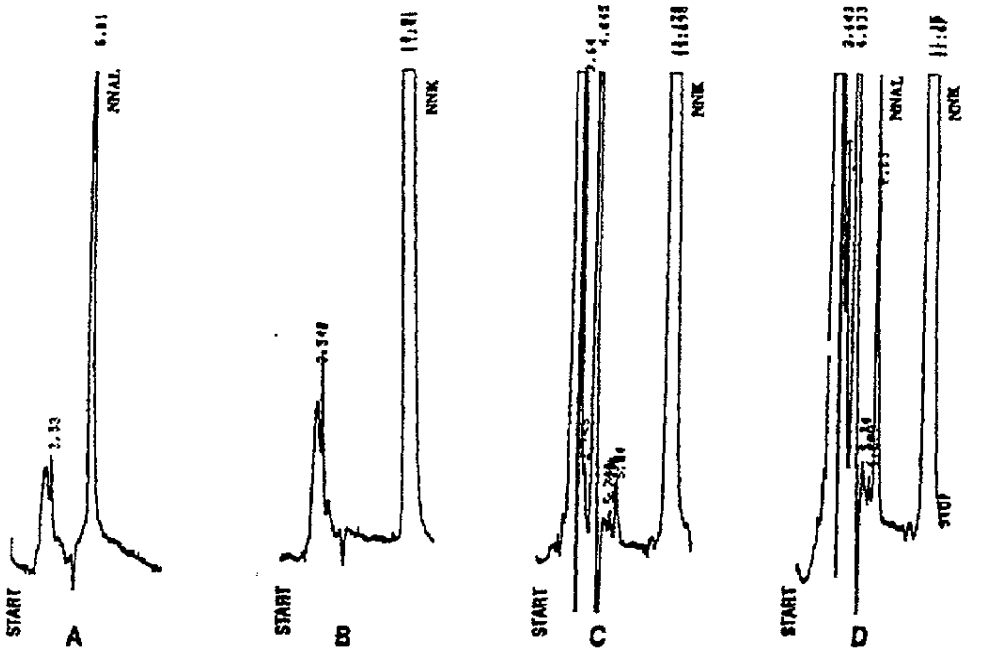

Fig. 2. HPLC chromatograms of authentic standard NNAL or NNK, or after a 60-min incubation of NNK in the absence or presence of purified 11β-HSD enzyme. 11.8 ng NNAL (A) or 103.6 ng NNK (B) as authentic standard substances, dissolved in HPLC eluent, were applied onto the HPLC system. NNAL elutes at about 6.8 min and NNK at 11.0 min. 100 μM NNK substrate was incubated for 60 min in the standard reaction mixture with a NADPH-regenerating system in the absence (C) or presence (D) of 5.2 μg purified 11β-HSD enzyme. Samples were processed as described in Materials and Methods. The formation of NNAL due to carbonyl reduction of NNK by 11β-HSD, becomes apparent (D). Peaks eluting in front of NNAL reflect components of the NADPH-regenerating system (C, D).

protein) and 10 μl NADPH-regenerating system (final concentrations: 0.8 mM $NADP^+$, 6 mM Glc6P, 0.35 U Glc6P dehydrogenase, 3 mM $MgCl_2$) in 10 mM sodium phosphate pH 7.4 at 37°C. The reaction was started by adding 10 μl NNK to final concentrations ranging over 0.05–5 mM. The reaction was stopped after 30 min by denaturating the protein for 2 min at 100°C before transferring the complete reaction mixture (50 μl) onto ice. After adding 150 μl ice-cold HPLC eluent (25% acetonitrile in 10 mM sodium phosphate pH 7.4), the samples were centrifuged at 8000×g for 6 min in the cold to sediment organic material; 20 μl of the supernatant was used for the HPLC detection of NNK and its carbonyl-reduced alcohol metabolite NNAL. Inhibitor studies were performed by adding 10 μl of a stock solution of glycyrrhetinic acid (final concentrations: 1–4 μM), corticosterone (final concentrations: 10–100 μM) or dehydrocorticosterone (final concentrations: 10–100 μM) to the incubation mixture. Inhibitors were dissolved in ethanol, yielding a final concentration of 2% which did not affect enzyme activity.

Control experiments without addition of 11β-HSD were performed to determine nonenzymatic substrate conversion. Specific activities are expressed as the rate of formation of NNAL/mass protein. Data were analyzed by Lineweaver-Burk and Dixon plot equations.

The oxidoreduction of glucocorticoids was determined as described elsewhere (Maser and Bannenberg, 1994a).

Immunoinhibition of 11β-HSD-mediated carbonyl reduction of NNK. For immunoinhibition studies, 10, 15 and 20 μl affinity purified antibodies (corresponding to final concentrations of about 2.3, 3.5 and 4.6 μg protein, respectively) were incubated with 5.2 μg purified 11β-HSD enzyme for 10 min before starting the reaction by simultaneously adding the substrate NNK (final concentration 100 μM) and the NADPH-regenerating system. Incubation conditions were the same as described above.

Determination of NNAL by HPLC. After enzymatic conversion, NNK and its reduced alcohol metabolite NNAL were detected on a BioRad reverse-phase HPLC system, using a LiChrosphere 100 RP18 column (4.6 mm×25 cm; Merck) and an eluent of 25% (by vol.) acetonitrile in 10 mM sodium phosphate pH 7.4. NNK elutes at about 11.0 min and NNAL at 6.8 min (flow rate 0.5 ml/min) and were monitored by absorbance at 230 nm with HPLC integration software. The identity of NNK and NNAL was determined by injecting authentic standards of both substances onto the HPLC system (Fig. 2). Concentrations of NNK and NNAL were calculated referring to corresponding calibration curves.

Determination of protein. Protein concentration was determined by the method of Lowry et al. (1951).

Kinetic parameter estimations. Time and enzyme protein concentratons were chosen so that reaction velocities were linear with time, which was found to be true at least for 1 h. The apparent kinetic parameters were determined by using the GraphPad Inplot kinetic computer software.

SDS/PAGE and immunoblotting. SDS/PAGE and immunoblotting were performed as previously described (Maser and Bannenberg, 1994b).

RESULTS

NNK carbonyl reduction by 11β-HSD. Our results demonstrate that purified 11β-HSD from mouse liver microsomes catalyzes the carbonyl reduction of the tobacco-specific nitrosamine NNK

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Table 1. Kinetic properties of purified 11β-HSD. Enzyme activities were assayed in the standard reaction mixture containing purified 11β-HSD, the indicated cosubstrate (R. S. = NADPH-regenerating system) and varying substrate concentrations (glucocorticoids: 0.125–0.4 mM; NNK: 0.05–5 mM). Kinetic parameter estimations were calculated from three experiments using the GraphPad Inplot kinetic computer software.

Substrate	Cosubstrate	V_{max}	K_m	V_{max}/K_m
		nmol · min⁻¹ · mg⁻¹	mM	
Corticosterone	$NADP^+$	329.33	0.66	498.48
Dehydrocorticosterone	NADPH	5.33	0.22	24.23
NNK	R.S.	135.13	1.75	77.22

Table 2. Inhibition of NNK carbonyl reduction by glucocorticoids and glycyrrhetinic acid. Enzyme activities were assayed in the standard reaction mixture containing purified 11β-HSD and an NADPH-regenerating system. To determine the kinetic parameters for inhibition of NNAL formation, three different concentrations of NNK (100–600 μM) were used in the presence of various inhibitor concentrations (glucocorticoids 0–100 μM; glycyrrhetinic acid 0–4 μM). K_i values were calculated by Dixon plot equations using the GraphPad Inplot kinetic computer software.

Inhibitor	K_i
	μM
Glyrrhetinic acid	1.52
Dehydrocorticosterone	16.31
Corticosterone	29.02

to its alcohol metabolite NNAL (Fig. 2). Table 1 shows the Michaelis-Menten kinetics of NNAL formation by 11β-HSD in the presence of a NADPH-regenerating system and substrate concentrations in the range 0.05–5 mM. For comparison, the values of glucocorticoid oxidoreduction by 11β-HSD, which have been determined in the presence of either $NADP^+$ or NADPH, are also given in Table 1.

Inhibition of NNK carbonyl reduction by glucocorticoids and glycyrrhetinic acid. Table 2 illustrates that the glucocorticoids corticosterone and dehydrocorticosterone, as well as the steroidal compound glycyrrhetinic acid, are potent inhibitors of NNK carbonyl reduction. Whereas the K_i values of NNK inhibition for glucocorticoids are significantly higher than those for glycyrrhetinic acid, the low K_i value of glycyrrhetinic acid corresponds well to those of this diagnostic 11β-HSD inhibitor obtained in other studies. The competitive nature of inhibition of NNK carbonyl reduction by glucocorticoids and glycyrrhetinic acid (data not shown) suggests that both steroids and NNK bind to the catalytically active site of 11β-HSD, and that NNK indeed is a substrate of this enzyme.

Inhibition of NNK carbonyl reduction by anti-(11β-HSD) antibodies. In order to conclusively substantiate the involvement of 11β-HSD in NNK carbonyl reduction, affinity purified antibodies against mouse liver 11β-HSD were employed to inhibit enzyme activity. Fig. 3 shows that the rate of NNAL formation by 11β-HSD was decreased by increasing amounts of the antibodies, leading, for example, to an inhibition of 71% when mixing about 4.6 μg antibody protein with about 5.2 μg 11β-HSD protein.

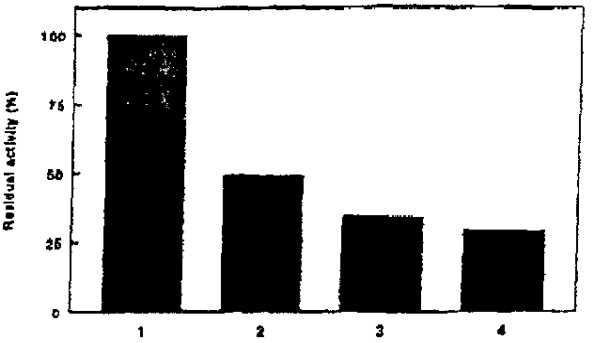

Fig. 3. Immunoinhibition of NNK carbonyl reduction by affinity purified antibodies against 11β-HSD. Antibodies (0, 2.3, 3.5 and 4.6 μg protein corresponding to columns 1, 2, 3 and 4, respectively) were mixed with 5.2 μg purified 11β-HSD for 10 min before starting the incubations. Values are means of two different preparations and are expressed as the percentage of residual enzyme activity compared to corresponding controls (column 1).

DISCUSSION

It is well established that carbonyl reduction to NNAL constitutes a major metabolic route of NNK clearance in laboratory animals and in many isolated experimental systems (Liu et al., 1990; Jorquera et al., 1992; Murphy et al., 1990; Schulze et al., 1995; Guo et al., 1992; Demkowicz-Dobrzanski and Castonguay, 1992; Morse et al., 1990b; Rossignol et al., 1989), accounting, under certain conditions, for 75% of the sum of all NNK metabolites formed (Murphy et al., 1990). Moreover, the actual rate of NNAL formation *in vivo* may be underestimated because NNAL has been shown to become conjugated with glucuronic acid to a significant extent *in vivo* (Murphy and Coletta, 1993; Morse et al., 1990a,b; Hecht et al., 1994; Carmella et al., 1993; Richter and Tricker, 1994; Schulze et al., 1992). In smokers, the level of NNAL glucuronide in 24-h urine accounted for 27–66% of their estimated daily dose of NNK (Carmella et al., 1993)

Although NNAL is somewhat less potent than NNK in terms of carcinogenicity (Liu et al., 1990; Castonguay et al., 1983), it should be noted that NNAL is not a non-toxic metabolite (Rivenson et al., 1988). NNAL induces tumors in the lung, liver and exocrine pancreas. NNAL may be responsible for NNK-induced pancreas tumors which were observed only after oral but not subcutaneous administration of NNK (Rivenson et al., 1988). DNA binding studies have shown that NNAL yields the same DNA adducts as those observed upon *in vivo* administration of NNK (Castonguay et al., 1983; Hecht and Trushin, 1988). Whether NNAL requires reconversion to NNK to gain its carcinogenic activity, or whether NNAL itself is α-carbon hydroxylated to reactive species that could damage DNA, remains in controversy (Murphy et al., 1995; Hecht and Trushin, 1988; Liu et al., 1990). However, in addition to pyridine-N-oxidation of both NNK and NNAL, the main detoxification pathway of NNK is conjugation of NNAL to glucuronic acid.

For more than a decade, the enzyme responsible for NNK carbonyl reduction has awaited its characterization (Hecht et al., 1980). In the present investigation we, finally, found that the microsomal enzyme which catalyzes the carbonyl reduction of NNK is identical to 11β-HSD, the physiological function of which is the oxidoreduction of glucocorticoids. Hence, 11β-HSD can be counted as initiating final detoxification of NNK, because 11β-HSD, by reducing the ketone moiety of NNK, provides the hydroxy function necessary for glucuronosylation.

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The genotoxicity of NNK has been considered to be dependent on the relationship between its metabolic activation by cytochrome P450 enzymes and constitutive DNA repair mechanisms (Pohlmann et al., 1992). In addition, the position of the NNK/NNAL balance in various tissues and species is likely to play a role in the organospecificity and carcinogenic potency of NNK. This balance is not only correlated with the distribution of 11β-HSD, but is also dependent on the co-expression of 11β-HSD and glucuronosyltransferase in a given tissue, since the glucuronosylation of NNAL formed by 11β-HSD results in a flux shift from NNK to NNAL.

Depending on the extent of the competing pathways, α-hydroxylation versus carbonyl reduction followed by glucuronosylation, the susceptibility of a tissue to NNK-induced tumorigenesis may be influenced. For example, the existence of low-K_m cytochrome P450 isozymes for NNK bioactivation in lung has been suggested to partially account for the higher susceptibility of the lung than the liver in NNK-induced tumorigenesis (Guo et al., 1992), whereas in liver itself (the organ with highest levels of 11β-HSD and glucuronosyltransferase), NNK has been found to be predominantly metabolized via carbonyl reduction and glucuronosylation, thus possibly explaining the lower tumor incidence of the liver after NNK exposure. The absence of NNAL formation in the nasal microsomes (Guo et al., 1992; Smith et al., 1992b; Hong et al., 1991; Brittebo et al., 1983) might also be a causative factor of the organospecific incidence of tumors in this tissue, a fact which might possibly be due to a lack of 11β-HSD expression in this tissue, in contrast to high levels of activating P450 isozymes (Hong et al., 1991; Guo et al., 1992; Smith et al., 1992b). In addition, the different extent of NNK carbonyl reduction by organs involved in the first-pass metabolism after subcutaneous (lung) or oral (small intestine, liver and lung) administration provides an explanation for the different tumor spectrum observed after NNK administration by either subcutaneous injection (lung, liver, nose) or in the drinking water (lung, liver, pancreas) (Hecht and Hoffmann, 1988; Rivenson et al., 1988). It is, therefore, of considerable importance to study the tissue distribution of NNK activating and inactivating enzymes in order to understand the biochemical mechanisms of NNK carcinogenesis.

The fact that a hydroxysteroid dehydrogenase, which is involved in the metabolism of endogenous steroids, is also responsible for the metabolism of a cancerogenic nitrosamine, is of particular interest in view of the role that this pluripotent enzyme has in regulating the actions of its physiological steroid substrates as well as in participating in the elimation of xenobiotics. For example, in response to ACTH secretion from the anterior pituitary gland during stress, elevated levels of endogenous glucocorticoids would occupy the 11β-HSD enzyme, resulting in a shift of the NNK/NNAL flux towards NNK, which, upon metabolic activation via α-carbon hydroxylation exerts its cancerogenic effect. It is also suggested that elevated levels of bile acids in cholestatic states might impair the activity of 11β-HSD (Bühler et al., 1994). Furthermore, several compounds of exogenous origin which are known to inhibit 11β-HSD could influence NNK carcinogenicity by modulating NNAL formation. This might be true for food constituents like glycyrrhetinic acid (Escher et al., 1995; Stewart et al., 1987) (a main constituent of licorice) and naringenin (Zhang et al., 1994) (a flavonoid with high concentrations in grapefruit juice), or pharmacologic substances such as the anti-ulcer drug carbenoxolone (Stewart et al., 1990) or the diuretic furosemide (Zhang et al., 1994; Escher et al., 1995). It might well be that smoking and the concurrent exposure to 11β-HSD modulators may have profound influences on the carcinogenic potency of NNK.

In summary, we give evidence that 11β-HSD from liver (11β-HSD1) mediates carbonyl reduction of NNK, and thus contributes to the final detoxification of this tobacco-specific nitrosamine by providing the base for its glucuronosylation. In the light of the known species and tissue differences in the expression of 11β-HSD isozymes, important aspects of NNK-induced tumorigenesis, such as metabolic activation versus inactivation or organospecificity, can now be re-evaluated.

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This work was supported by a grant from the Alfred und Ursula Kulemann-Stiftung, Marburg.

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